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Fungi and insects associated with *Euphorbia ingens* die-off in South Africa

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***Euphorbia ingens,* landmark succulent trees in savannas of South Africa, have been dying in large numbers over the last 10–15 years. Initial studies conducted in the Limpopo province of South Africa revealed a diverse group of biotic agents including fungi, beetles and moths associated with dying trees, but due to the limited geographic extent of these studies, it was not known if the same agents were associated with dying trees regionally. In this study, diseased and insect-infested trees were sampled for fungal pathogens and insects at six sites in four provinces located across South Africa. Fungi were identified based on morphology and DNA sequencing of the ITS, LSU, β-tubulin and TEF 1-α gene regions, and insects were identified based on morphology. Fungal isolates were identified as *Aureovirgo volantis*, *Fusarium solani*, *Lasiodiplodia* × *egyptiacae*, *Ophiostoma thermarum* and a *Readeriella* species. Five insects were identified, all in the family Curculionidae, including two ambrosia beetles, *Cyrtogenius africus* and a *Stenoscelis* species. All fungi and insects collected are known to be opportunistic and occur on stressed trees as secondary agents of mortality or disease. These results suggest that the die-off is not related to attack of the trees by aggressive insects or pathogens, but rather that *E. ingens* in this region is under stress from environmental factors that supports the ability of opportunistic insects and pathogens to establish.**

Keywords: ambrosia beetles, opportunistic pathogens, tree stress

Introduction

Euphorbia ingens (common names include giant euphorbia tree, candelabra tree and naboom) are dying at a rapid rate in some regions of South Africa. The first reports of large-scale *E. ingens* mortality were from the Limpopo province. Causes of mortality were speculated to be from stress due to climate change or infestation by invasive insects or pathogens (Malan 2006; Roux et al. 2008, 2009). A subsequent study, comparing mortality at sites in the Limpopo and North West provinces, indicated that die-off was most severe in the Limpopo region (van der Linde et al. 2012) and was most likely related to changes in temperature and rainfall patterns that contributed to insect attack and disease development (van der Linde et al. 2012). Additional studies implicated several fungal and insect agents as possible causes of tree mortality (Roux et al. 2008, 2009; van der Linde et al. 2011a, 2011b). These studies suggested that *E. ingens* die-off may be the result of environmental factors that create stress in the trees, leading to attack by opportunistic insects and pathogens (van der Linde et al. 2011a, 2011b, 2012). However, given the limited geographic extent of the initial studies, surveys made across a broader area were needed to know if this was indeed the case.

The aim of this study was to conduct surveys assessing symptoms associated with the die-off and associated insects and fungi across the range of *E. ingens* in South Africa. Furthermore, we wished to determine if any of these biotic agents were consistently associated with trees in areas experiencing die-off.

Materials and methods

Estimation of mortality and disease symptoms associated with E. ingens *die-off*

In 2014, disease symptoms and mortality in declining *E. ingens* stands were scored at nine sites (Figure 1) across South Africa, including five sites previously investigated by van der Linde et al. (2012) in 2009 and 2010. Eight belt transects of 100 m × 50 m were established at each site and their location recorded using a global positioning system (GPS). Based on van der Linde et al. (2012), two specific symptoms were evaluated for individual *E. ingens* trees: grey discoloration and rotting of succulent branches.

Grey discoloration and moth damage were scored, independently from one another, based on a ranking system of zero (no grey discoloration or moth damage) to four (1: 1–25% succulent branches grey and rotting from moth damage, 2: 26–50%, 3: 51–75%, 4: 76–100%). Grey discoloration and the rotting of succulent branches affect *E. ingens* trees differently, and even with cases where the two symptoms occur on the same tree, they do not typically occur on the same branch. The moths attack succulent

Figure 1: Sites at which insect and fungal surveys and die-off symptom scoring were conducted. 1 = Enzelsberg, 2 = Wolfaan, 3 = Bela-Bela, 4 = Lydenburg; 5 = Ulundi, 6 = Eshowe, 7 = Euphorbia Drive, 8 = Capricorn, 9 = Last Post

branches randomly with no clear pattern of rotting, hence the succulent crown was visualised as a quadrant with succulent branches scored accordingly. Grey discoloration was easier to score, occurring as a gradual progression starting at the lower ends of the succulent branches just above the main trunk.

Mortality was scored as a percentage of dead trees in each transect compared with living trees. The mean rank proportion of grey and moth-damaged trees, as well as percentage mortality, was calculated (data were tested for normality using Shapiro–Wilk's *W*) and compared among the nine sites using analysis of variance (ANOVA). Mean separation analyses were conducted using Tukey–Kramer's test. Linear regression analysis, among all sites, was conducted to test if mortality was dependent on moth damage and/or grey discoloration. All statistical analyses were conducted using JMP 12.0.1 (SAS Institute, Cary, NC, USA, 1989–2007) with $\alpha \leq 0.05$.

Fungus and insect collections

Surveys of diseased *E. ingens* were conducted in 2012 and 2013 at six sites with one inspection conducted at each site per year (Figure 1; Sites 1 to 6). The same area, within which the belt transects were established for the symptom and mortality scoring survey, was used for the surveys at each site. At each site, one branch exhibiting each symptom type (grey discoloration of the succulent branches, rotting of the succulent branches surrounding moth damaged areas, or staining in the main woody stems associated with insect infestation) was collected from 10 different trees for each symptom. Symptomatic tissue samples were placed in paper, and/or plastic bags and transported to the laboratory for further investigation.

Isolations for fungi were made by surface disinfesting plant tissue and cutting small segments from the leading edges of diseased areas and transferring the tissue to 2% malt extract agar (MEA; 15 g agar and 20 g malt extract L^{-1}; Biolab, Merck, Midrand, South Africa) amended with streptomycin (0.4 g L^{-1}; Sigma-Aldrich, St Louis, MO, USA). When fungal fruiting bodies were present on lesions or in insect tunnels, spore drops and/or hyphae were carefully removed from the plant material using a sterilised needle and placed on 2% MEA plates. The resultant colonies from tissues and fungal material were purified using single spore or hyphal tip transfers onto 2% MEA plates. After 5 d of growth, cultures were grouped according to each disease symptom and then further grouped based on the most commonly occurring pure cultures. Representatives from each morphological group were sequenced (using the ITS, LSU, β-tubulin and TEF 1-α gene regions) and identified to genus and, where possible, species level. Representative isolates have been deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa.

Insects associated with diseased trees were obtained from freshly infested branches and stems by collecting 10 logs from 10 different trees from each site within the already established transect areas. Collections were made during March 2013 and 2014. For each site, four logs were placed in four emergence chambers, which were monitored daily for insect emergence over a period of two weeks. Logs could not be kept for a longer period within the emergence chambers as *E. ingens* branches and stems rot and disintegrate very quickly due to their high moisture content. The remaining six logs, from each site, were dissected

in the laboratory and insects collected pre-emergence. Insects collected from emergence chambers and dissected logs were grouped based on morphology using the keys in Wood (1986), and counts made for each group. Insects were identified by Dr Roger Beaver (Thailand). Representative specimens of beetles were pinned and deposited with the National Collection of Insects, Plant Protection Research Institute, Agricultural Research Council, Roodeplaat, Pretoria, South Africa as well as the collection of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Results

Estimation of mortality and disease symptoms associated with E. ingens *die-off*

Symptoms associated with die-off were present at all sites investigated and at varying levels of severity (Table 1). There was a significant difference in severity of the two main die-off symptoms (greying and rotting) as well as in the percentage mortality among the sites (Table 1). The sites with the highest proportion of rotting (moth damage) were Enzelsberg, Wolfaan, Ulundi followed by Bela-Bela, Euphorbia Drive, Last Post and Capricorn, Eshowe and Lydenburg. The sites with the highest proportion of grey discoloration were Euphorbia Drive, Last Post, and Ulundi followed by Capricorn, Eshowe, Lydenburg, Bela-Bela, Enzelsberg, and Wolfaan. Overall, the most severely affected sites (highest mortality) were Enzelsberg, Euphorbia Drive and Last Post with the least affected sites being Capricorn, Eshowe and Lydenburg.

Sites with the highest mean rank greying did not always have the highest percentage of mortality of *E. ingens* and does not seem to be correlated ($R^2 = 0.01$, $P = 0.3180$). Euphorbia Drive, Last Post and Ulundi exhibited the highest mean rank greying with a correspondingly high percentage of mortality, while Capricorn had a high mean rank of grey discoloration with the lowest percentage mortality. Enzelsberg had the highest percentage mortality but the lowest mean rank of greying among all the sites. Moth damage was correlated ($R^2 = 0.212$, $P < 0.001$) with higher percentage mortality, with the sites with the highest degree of die-off having higher levels of moth-related damage.

Fungus and insect collections

Isolations from diseased tissue yielded a total of 351 isolates for the six sites, with most isolates being saprophytes such as *Penicillium* species. From the 351 isolates, 100 were identified as the most consistently associated with the observed disease symptoms (Table 2). The isolates were divided into three main groups based on morphology. Representative isolates (Table 3) from each morpho-group were further identified using DNA sequence analysis, from which five genera were identified (Table 3).

Based on DNA sequence data, isolates were identified as *Aureovirgo volantis* (TreeBase: 17782, 17783) described previously by van der Linde et al. (2016) from *E. ingens*, an undescribed *Fusarium* sp. (TreeBase: 17784, 17785) in the *Fusarium solani* species complex, *Lasiodiplodia* × *egyptiacae* (TreeBase: 17788, 17789) (recently identified as a hybrid of *L. theobromae* and possibly *L. parva* or *L. citricola*; Cruywagen et al. 2016), *Ophiostoma thermarum* (TreeBase: 17782, 17783) described previously by van der Linde et al. (2016) from *E. ingens*, and an apparently undescribed *Readeriella* sp. (TreeBase: 17786, 17787). *Lasiodiplodia* × *egyptiacae* and *F. solani* were isolated from stained areas of the main stems of trees heavily infested with weevils as well as rotted tissues associated with moth damage. *Readeriella* sp. was isolated from fruiting bodies in grey as well as green succulent areas on the outside of the branches. *Aureovirgo volantis* and *O. thermarum* were commonly found within the tunnels of the ambrosia beetles *Cyrtogenius africus* and *Stenoscelis* sp., in succulent branches and the sapwood of the main stems (Figure 2).

Fungal isolations were successful from only 55 (out of the 180 collected) branches (each branch from a different tree, $N = 55$ trees). Most of the isolates obtained were associated with insect damage (rotting associated with moth attacks and staining associated with weevil attacks) with only six isolates from greyed areas. Isolates associated with insect damage were obtained from all of the sites, while isolates from the grey discoloured tissue were obtained from only two sites (Table 2).

Five Curculionidae species (two ambrosia beetles and three weevils) were collected from the emergence chambers (Table 2). The ambrosia beetles (Scolytinae), *Cyrtogenius africus* (AcP9546) and a *Stenoscelis* sp. (AcP9549), were reared from the main stems, whereas

Table 1: Mean die-off factor and percentage mortality of *Euphorbia ingens* at nine sites and one-way ANOVA statistics of comparisons among sites for each factor and mortality. Values in parentheses are the SE. The same letter within a column indicates that means are not significantly different ($\alpha \leq 0.05$)

Site	Grey discoloration	Moth damage	Mortality (%)
Enzelsberg	0.148 (0.056)^{c}	0.689 (0.120)^{ab}	32.50 (3.694)^{a}
Euphorbia Drive	1.695 (0.073)^{a}	0.597 (0.054)^{ab}	25.52 (5.102)^{ab}
Last Post	1.407 (0.062)^{a}	0.584 (0.047)^{ab}	20.90 (4.454)^{abc}
Ulundi	1.659 (0.083)^{a}	0.775 (0.047)^{a}	17.47 (5.889)^{abcd}
Wolfaan	0.351 (0.044)^{bc}	0.774 (0.080)^{a}	16.40 (4.061)^{abcd}
Bela-Bela	0.560 (0.071)^{b}	0.504 (0.092)^{ab}	14.90 (2.039)^{bcd}
Eshowe	0.657 (0.118)^{b}	0.406 (0.068)^{bc}	10.62 (3.196)^{bcd}
Lydenburg	0.594 (0.141)^{b}	0.166 (0.048)^{c}	7.00 (2.479)^{cd}
Capricorn	0.739 (0.082)^{b}	0.170 (0.026)^{c}	2.50 (1.732)^{d}
ANOVA statistics	$F = 43.847$, df = 8, $P < 0.001$	$F = 10.863$, df = 8, $P < 0.001$	$F = 5.702$, df = 8, $P < 0.001$

Table 2: Number of isolates obtained from isolations from dying *Euphorbia ingens* trees exhibiting the three main symptoms of disease at six sites in South Africa

	Disease symptom[a]	Enzelsberg	Wolfaan	Lydenburg	Bela-Bela	Ulundi	Eshowe	Total isolates
Fungal species[b]								
Fusarium solani	Rotting of succulent branch	3 [2]	–	27 [7]	5 [2]	2 [1]	2 [1]	39
Lasiodiplodia × *egyptiacae*	Rotting of succulent branch	–	12 [4]	–	–	3 [3]	–	15
Readeriella sp. nov.	Grey discoloration	–	–	2 [2]	–	–	4 [4]	6
Ophiostoma thermarum	Stain/galleries in main woody stem	–	–	–	16 [7]	–	–	16
Aureovirgo volantis	Stain/galleries in main woody stem	6 [6]	4 [4]	7 [7]	4 [3]	–	3 [3]	24
Insect presence[c]								
Megasis sp.		+	+	+	+	+	+	
Cossonus sp.		–	+ (32)	–	–	+ (22)	–	
Mechistocerus sp.		+ (5)	+(15)	–	–	–	–	
Stenoscelis sp.		+ (105)	+ (53)	+ (23)	+ (45)	–	–	
Coleobothrus germeauxi		+ (163)	+ (180)	–	–	–	–	
Cyrtogenius africus		+ (90)	+ (145)	+ (41)	+ (28)	–	+ (38)	

[a] 10 branches from 10 trees were collected for each disease symptom

[b] Values are the number of isolates [no of branches]

[c] Values are the number of beetles that emerged from rearing containers

Table 3: Genbank accession numbers and locality of collection of representative isolates sequenced and identified in this study

Species	CMW	Locality	ITS	TEF 1-α	LSU	β-tubulin
Fusarium solani	–	Enzelsberg	KU519629	KU519634	–	–
F. solani	–	Lydenburg	KU519630	KU519635	–	–
F. solani	–	Bela-Bela	KU519631	KU519636	–	–
F. solani	–	Ulundi	KU519632	KU519637	–	–
F. solani	–	Eshowe	KU519633	KU519638	–	–
Lasiodiplodia × *egyptiaceae*	38914	Wolfaan	KU519639	KU519643	–	–
L. × *egyptiacae*	38915	Wolfaan	KU519640	KU519644	–	–
L. × *egyptiacae*	38916	Wolfaan	KU519641	KU519645	–	–
L. × *egyptiacae*	38917	Ulundi	KU519642	KU519646	–	–
Ophiostoma thermarum	38929	Bela-Bela	KR051114	–	KR051126	KR51102
O. thermarum	38930	Bela-Bela	KR051115	–	KR051127	KR51103
O. thermarum	38931	Bela-Bela	KR051116	–	KR051128	KR51104
Aureovirgo volantis	42282	Eshowe	KR051123	–	KR051133	KR51109
A. volantis	42285	Lydenburg	KR051121	–	KR051134	KR51110
A. volantis	42287	Bela-Bela	KR051124	–	KR051135	KR51111
A. volantis	42290	Enzelsberg	KR051122	–	KR051136	KR51112
A. volantis	42292	Wolfaan	KR051125	–	KR051137	KR51113
Readeriella sp.	44675	Eshowe	KU519647	KU519649	–	–
Readeriella sp.	44676	Lydenburg	KU519648	KU519650	–	–

two weevils, *Mechistocerus* sp. (Molytinae) (AcP9551) and *Coleobothrus germeauxi* (Scolytinae) (AcP9544), were reared from the secondary phloem. The weevil *Cossonus* sp. (Cossoninae) (AcP9548) was reared from the vascular cambium (Figure 3). Larvae of the moth *Megasis* sp. (Lepidoptera: Pyralidae) were identified at all sites and were associated with the rotting of the succulent branches.

Discussion

The results of this study expand on those of Roux et al. (2008, 2009) and van der Linde et al. (2011a, 2011b, 2016) who reported a number of fungi and insects associated with the large-scale die-off of *E. ingens* in the Limpopo province of South Africa. Sites for the present study were selected over a wider geographic distribution of *E. ingens* in the country, allowing a more comprehensive evaluation of the factors associated with the die-off. There were differences in severity of symptoms associated with die-off and mortality among the sites. Higher levels of moth damage were observed at sites with higher tree mortality. *Megasis* sp. occurred at all sites, suggesting a stronger correlation between tree death and infestation by this moth compared with grey discoloration. Isolations from grey discoloured branches yielded very few fungal isolates and the grey discoloration of the branches is not caused by fungal infections.

Relatively few fungal isolates were obtained from diseased material sampled in this study, despite the large number of samples collected. These results are similar to previous studies by van der Linde et al. (2011a, 2011b)

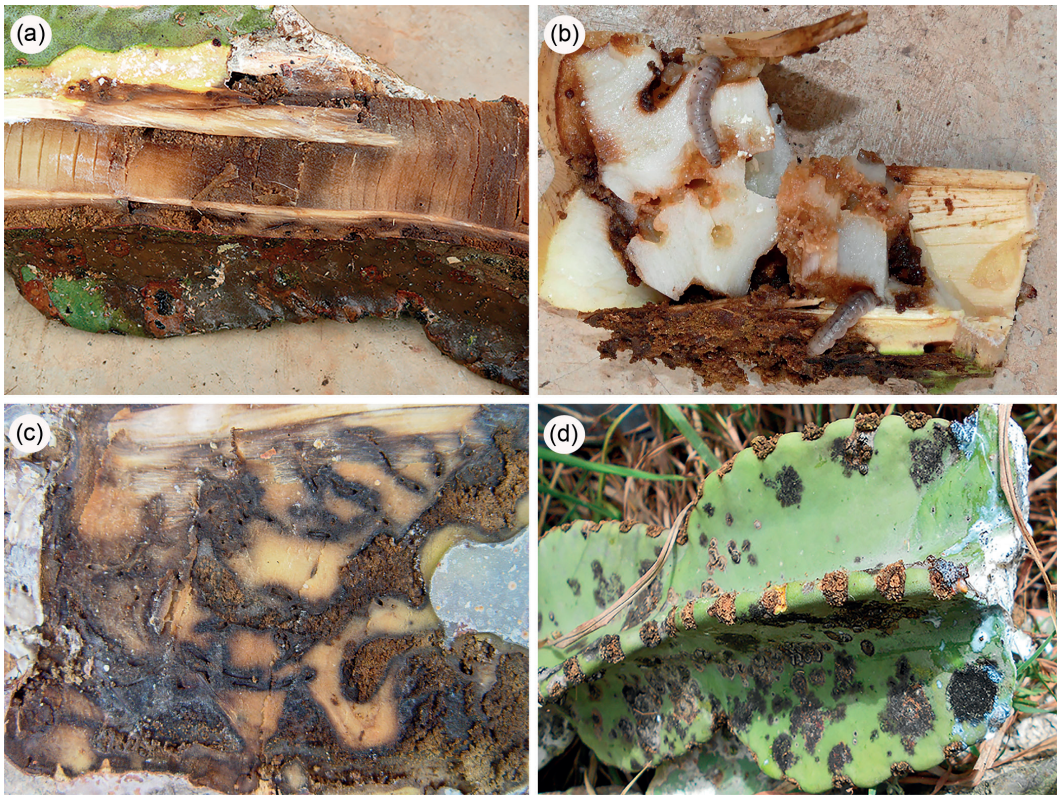

Figure 2: Symptoms of disease and insect infestation associated with *Euphorbia ingens* die-off. (a) Moth-attacked branch from which *Lasiodiplodia* × *egyptiacae* and *Fusarium solani* were isolated, (b) larvae and damage caused by *Megasis* sp., (c) staining associated with beetle tunnelling from which *Aureovirgo volantis* and *Ophiostoma thermarum* were isolated, (d) black fruiting bodies of *Readeriella* sp. on succulent branches

and may either be a result of low isolation success and/or an indication of the secondary nature of fungal involvement in the death of *E. ingens*. Of the isolates obtained, only a few represented possible pathogens. Of these, *A. volantis*, *F. solani*, *L.* × *egyptiacae* and *O. thermarum* were most often isolated from *E. ingens* trees that were heavily infested by the moth *Megasis* sp. and ambrosia beetles (*C. africus* and *Stenoscelis* sp.). *Lasiodiplodia* species (Botryosphaeriaceae) are well-known opportunistic fungal pathogens known to cause staining within wood, dieback and cankers of stressed trees and are associated with a wide variety of hosts (Damm et al. 2007; Slippers and Wingfield 2007; Phillips et al. 2008; Jami et al. 2015). Species in this genus, such as *L. theobromae* and *L. mahajangana,* have been reported previously from dying *E. ingens* trees (van der Linde et al. 2011b) and are also known from *Acacia*, *Eucalyptus*, *Pinus* and native *Syzygium* in South Africa (Crous et al. 2000; Burgess et al. 2003; Pavlic et al. 2004, 2007). *Lasiodiplodia* × *egyptiacae* was described from mango (*Mangifera indica*) plantations in Egypt (Ismail et al. 2012) and has been reported from physic nut (*Jatropha curcas*) in Brazil as well as baobabs (*Adansonia grandidieri*) in Madagascar (Machado et al. 2014; Cruywagen et al. 2016).

Aureovirgo volantis and *O. thermarum* are members of the *Ophiostoma sensu lato* complex (Ophiostomataceae). *Ophiostoma thermarum* resides in the *Sporothrix schenckii–Ophiostoma stenoceras* complex, a group of fungi known to be associated with soil and hardwoods as well as with conifer-infesting bark beetles (Zhou et al. 2001; de Beer et al. 2003; de Meyer et al. 2008; Roets et al. 2008). Even though fungi in this complex are known to cause staining in wood, their pathogenicity in host trees has been questioned, and they are considered as secondary agents to tree disease and mortality (de Beer et al. 2003; de Meyer et al. 2008). Species within this complex, in South Africa, have also been recorded from *Protea* species, *Eucalyptus grandis* and pine-infesting bark beetles (Wingfield et al. 1993; Zhou et al. 2001, 2006; Roets et al. 2008). Pathogenicity trials, conducted by van der Linde et al. (2016) using *A. volantis* and *O. thermarum* on *E. ingens*, produced small lesions and internal rotting on succulent branches (van der Linde et al. 2016). Van der Linde et al. (2016) did not find that *A. volantis* and *O. thermarum* are primary pathogens of *E. ingens*. Given that they are not known to be virulent pathogens, the species isolated in this study are unlikely to be major drivers of *E. ingens* die-off.

The *Fusarium solani* species complex (FSSC) comprises at least 45 closely related species (Zhang et al. 2006). The FSSC fungi are known to be soil borne or to occur in decaying organic material (Zhang et al. 2006; Bogale et al. 2009). Species in this group have been isolated from soil and lesions on a wide variety of crops, including potato, tomato, citrus, pea and soybean (Roy et al. 1989; Cho et al. 2001; Romberg and Davis 2007; Zaccardelli et al. 2008; Rehman et al. 2012). In South Africa, *F. solani* has been reported to cause the die-back of English walnut (*Juglans*

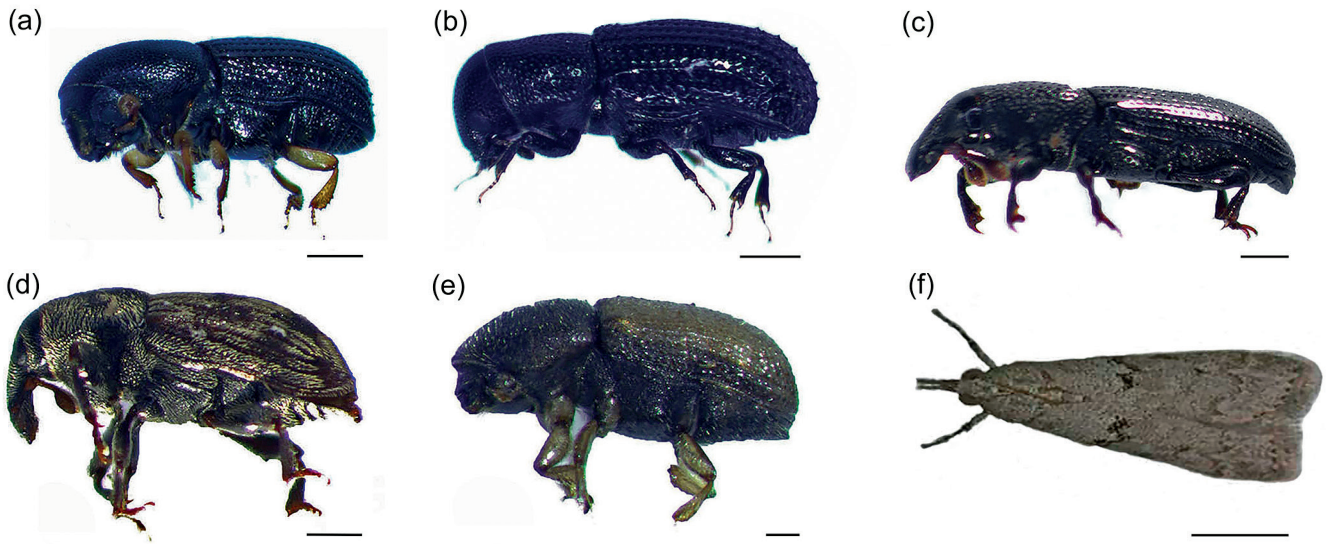

Figure 3: Insects identified from diseased *Euphorbia ingens* trees. (a) *Cyrtogenius africus* (sapwood), (b) *Stenoscelis* sp. (sapwood), (c) *Cossonus* sp. (vascular cambium), (d) *Mechistocerus* sp. (secondary phloem), (e) *Coleobothrus germeauxi* (secondary phloem), (f) adult *Megasis* sp. (secondary phloem). Scale bars: (a–d) 500 μm, (e) 200 μm and (f) 5 mm

regia) and lisianthus (*Eustoma grandiflorum*) (Chen and Swart 2000; Truter and Wehner 2004). Species within FSSC are known to be associated with ambrosia beetles (Baker and Norris 1968; Windels et al. 1976; Beaver 1989; Rojas et al. 1999; Mendel et al. 2012) and in some cases occur as mutualists, e.g. of *Hypothenemus hampei* (coffee borer beetle) (Rojas et al. 1999; Morales-Ramos et al. 2000). It has been suggested that these fungi could be opportunist pathogens causing disease and death in stressed plants (Sherbakoff 1953; Kavroulakis et al. 2007; Bogale et al. 2009; Rehman et al. 2012). In the present study, isolates of *F. solani* were consistently isolated from disease margins of rotten succulent branches, which had been fed on by *Megasis* larvae and infested by weevils. It seems unlikely that this fungus would be the primary cause of tree die-off and is more likely a secondary agent of disease.

The fungal genus *Readeriella* belongs to the family Teratosphaeriaceae, a well-known group of fungi that causes diseases of the stems and leaves of *Eucalyptus* species (Crous 1998; Crous et al. 2004; Burgess et al. 2007; Carnegie 2007; Cheewangkoon et al. 2008, 2009; Hunter et al. 2011). This is the first report of a *Readeriella* species from *E. ingens* trees. The fungus was commonly isolated from black fruiting bodies on the outside of the grey discoloured and green succulent branches of *E. ingens*. The black fruiting bodies only occurred on the exterior of the branches in a very superficial manner, never extending into the tissue.

The insects reared from diseased and dying *E. ingens* trees have all previously been reported from Africa. *Coleobothrus germeauxi* is known to occur in dead branches of trees and has been recorded from *Euphorbia teke* Schweinf. ex Pax in Kenya and Uganda (Jordal and Hewitt 2004; Mandelshtam and Danielsson 2004), while *Mechistocerus* sp. has only one record from Africa, namely from Liberia (Briscoe 1947). *Stenoscelis* species are known to attack trees that are stressed, with recorded collections from Algeria, Kenya and South Africa (Konishi 1956). *Cossonus* species have been reported from decaying trees in Ethiopia and the KwaZulu-Natal province in South Africa (Marshall 1905; Colonnelli 2014). *Cyrtogenius africus* was first recorded in 1988 from various *Euphorbia* species in Africa (Democratic Republic of the Congo [formally known as Zaire from 1971–1997], Guinea, Kenya, Tanzania, Uganda) and again in 2009 from dead branches of *Euphorbia triangularis* Desf. in South Africa (Wood and Bright 1992; Jordal 2009). However, there is limited information on how and when these beetles infest trees, with the only records existing being for diseased trees or decaying wood (Marshall 1905; Briscoe 1947; Konishi 1956; Wood and Bright 1992; Jordal and Hewitt 2004; Mandelshtam and Danielsson 2004; Jordal 2009; Colonnelli 2014). The beetles appear to be secondary, infesting stressed trees (Konishi 1956; Jordal 2009). Limited information is also available for host preferences and distribution of the moth, *Megasis* sp. Our specimens could only be identified to genus and are believed to be a species native to South Africa (Martin Kruger, The Ditsong National Museum of Natural History, Pretoria, South Africa, pers. comm.).

Van der Linde et al. (2012) found that *E. ingens* mortality was related to a specific province with temperature and rainfall having a significant effect on tree mortality. Temperature over the last 60 years has increased more significantly in the Limpopo province compared with the North West province and was identified as the main trigger for *E. ingens* die-off (van der Linde et al. 2012). However, in this study, sites within a province had different levels of tree mortality. Euphorbia Drive and Last Post had higher tree mortality compared with Bela-Bela and Capricorn, with Last Post having significantly higher percentage mortality compared with Capricorn (two sites that are 18 km from each other). Wolfaan had half the percentage mortality compared with Enzelsberg (North West), while Eshowe had lower percentage mortality compared with Ulundi (KwaZulu-Natal). Percentage mortality, therefore, does

not seem to be related to a specific province or area, as previously believed, and might be affected, not only by climate, but by more site-specific conditions as suggested by van der Linde et al. (2017).

Conclusions

It is known that trees in disturbed environments can be under substantial stress leading to susceptibility to secondary fungi and insects that can lead to the death of the trees (Mueller-Dombois et al. 1983; Akashi and Mueller-Dombois 1995; Dale et al. 2000; Holdenrieder et al. 2004; Foden et al. 2007; Allen 2009; Allen et al. 2010). Our results lead us to believe that *E. ingens* die-off is likely driven by stressors in the environment that lead to attack by insects and infection by pathogens that ultimately kill the tree. In order to understand the 'triggers' for *E. ingens* die-off, it will be necessary to study the environment in which these trees occur more closely. Such studies should then consider all of the abiotic factors, analysing the system as a whole together with the accompanying biotic factors.

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